PRODUCING CONTINUOUSLY GROWING DUCK AND AVIAN CELL LINES FROM PRIMAY AVIAN CELLS

A Senior Scholars Thesis

by

ZACHARY DEAN FRANKLIN

Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

May 2012

Major: Biomedical Science

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Approved by:

Research Advisor: Judith Ball
Associate Director, Honors and Undergraduate Research: Duncan MacKenzie

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ABSTRACT

Continuously Growing Duck and Avian Cell Lines derived from Primary Avian Cells.
(May 2012)

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The need for a continuous avian cell line is increasing in order to grow and study important avian viruses. The study of the virus responsible for proventricular dilatation disease (PDD), a bornavirus, would benefit from an available continuously-growing psitticine cell line. To attempt to Primary cells from cockatiel and duck embryos were prepared from unhatched cockatiel and duck eggs. They were expanded and frozen for storage and further use and evaluation. To transform the primary cells and generate a continuous cell line, the cells were grown in 48-well plates and treated with the carcinogen, 3-methylcholanthrene, at varying concentrations and times. Upon addition of the carcinogen some cells began to show morphological changes and in a few wells giant cells appeared. Some cells took on a cuboidal shape, while others piled on top of each other into circular clumps. To date these transformations have not proved to produce a continuous cell line.

DEDICATION

I would like to dedicate this paper to all the people in the world who are struggling to put themselves through college. These driven people start out more underprivileged than the majority, but decide to embark on the journey anyway, knowing it will be a tough road. I would like for them to know that when given the opportunity and by putting up a good fight they can succeed without the privileges that many others start with.

ACKNOWLEDGMENTS

First and foremost, I would like to think Dr. Judith Ball for allowing me to work in her lab. I have picked up a wide range of skills while doing research in her lab and have grown very fond of biomedical research in the process. I would like to thank her for allowing a collaborative project with Dr. Tizard, and I would like to thank Dr. Tizard for his help and guidance through the project. I am now very proficient and comfortable working with cell culture, which is a difficult skill to obtain.

I appreciate Krystle Yakshe and Joe Guo for working very closely with me and guiding me in my research. Both of them played a major role in mentoring me along the way. I would like to thank Deborah Turner for assisting me in learning how to harvest the embryo fibroblasts. Lastly, I would like to thank all of the staff in the VTPB department for helping me with the many tedious parts of getting through my degree.

NOMENCLATURE

ABV	Avian Bornavirus
BDV	Borna Disease Virus
CPE	Cytopathic Effect
CTEF	Cockatiel Embryo Fibroblast
DEF	Duck Embryo Fibroblast
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic Acid
FBS	Fetal Bovine Serum
G	Glycoprotien
L	L-Polymerase
M	Matrix Protein
MEM	Modified Eagle's Medium
MG	Milligram
ML	Milliliter
N	Nucleocapsid Protein Phosphate Buffered Saline
NEAA	Non-Essential Amino Acid
NES	Nuclear Exit Signal
NLS	Nuclear Localization Signal
P	Phosphoprotein

PDD	Proventriculus Dilation Disease
RPM	Revolution Per Minute
SV40	Simian Virus 40
3-MCA	3-Metylcholanthrene
UL	Microliter
X	Protein Unknown

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CHAPTER I

INTRODUCTION

The goal to establish avian cell lines is based on the need for avian cells to grow and study the important avian viruses. Producing avian cell lines has proven difficult and for the most part are unavailable. We particularly are interested in growing the virus responsible for Proventricular dilatation disease (PDD), a bornavirus.

PDD was discovered in 1977 by Stoddard and initially was named macaw wasting syndrome(8). It was first noticed in macaws because they lost weight, displayed a distended belly, and showed neurologic symptoms. When the macaws died, they were found to have a proventriculus, the first part of a bird's stomach, filled with undigested food, which caused it to increase dramatically in size(3). It was then found in 2008 that PDD was caused by a virus of the bornavirus family, and has since been named avian bornavirus (ABV)(4). ABV was thought to only infect psittacines until very recently when ABV was detected in ducks and Canadian geese by scientist at TAMU personal communications. These new viral strains, as well as those from parrot and cockatiels, can grow in duck embryo fibroblasts (DEFs), but these cells only undergo a few passages before entering senescence. The growth of the virus is difficult to study when having to constantly generate new host cells, which often display varying properties. We propose to create an immortalized cockatiel cell line

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This thesis follows the style *Journal of Virology*.

and an immortalized duck embryo fibroblast line, which will be the first of its kind, specifically for the propagation of and study of psittacine bornaviruses.

Bornaviruses from the family Bornaviridae are relatively simple viruses consisting of six proteins. They are a (N) nucleocapsid protein, (X) protein-unknown, (P) phosphoprotein, (L) L-polymerase, (M) Matrix protein, and (G) L glycoprotein. Its genome is a single stranded, negative sense RNA. The virus is highly cell associated, and causes no visual cytopathic effect (CPE) in infected cell cultures. Due to the virus tight cell association, in order to infect uninfected cells, one must add infected cells, not virus alone to accomplish virus propagation. The virus is usually not found in the culture media at high levels.

Since ABV is a newly described virus and there are no consistent avian cell lines to study the course of infection, we must use what is known about mammalian bornaviruses as a model of infection. Mammalian bornavirus enters the cell through an unknown receptor followed by receptor-mediated endocytosis. Once infected, the cells do not show signs of diminished growth or any other type of cellular disruption. Bornavirus can infect many different cell types in culture but in vivo it tends to only affect neuronal cells. If the virus is left to dry, it remains infectious for a long period of time.

Bornavirus is the only known animal negative-strand RNA virus with a nuclear phase. During the nuclear phase, bornavirus use alternative splicing to process the viral RNA. The nuclear localization signal (NLS) located in proteins N, P, and X, and the nuclear exit signal

(NES) in N and X, have elicited the development of a hypothesis that involves nucleocytoplasmic shuttling of mammalian bornavirus, but conclusive data are lacking. Budding of virion structures was observed in persistently infected cultured mammalian cells, and it is known that accurate processing and expression of the G glycoprotein are prerequisites for viral spread in tissue culture. (5)

Phylogenic analyses failed to reveal compelling similarities between BDV and other viruses (5). Phylogenetic analyses of the L sequence show a relationship between BDV, rhabadoviruses and paramyxoviruses, all of which are negative strand viruses. The BDV N-terminal half of the sequence is more closely related to rhabdoviruses while the C-terminal half more closely related to paramyxoviruses e (5). There is little known about avian Bornavirus strain variations. Without a continuous, stable cell line, it is difficult to study the dynamics of avian bornavirus infection and to explore possible interventions.

Currently, there exist a few chicken cell lines but most strains of avian bornavirus do not grow well in cultured chicken cells (1). A duck embryo fibroblast line is sufficient for virus growth, but a psittacine line is preferred so that the infection will mimic the natural infection as closely as possible. We initially will utilize the duck embryo fibroblast to establish conditions for primary avian cells. If we are unable to generate a psittacines cell line, we will utilize the duck embryo cells.

Cell culture was officially introduced by publication in the early 20th century, although it had been around prior to that time (4). It was well known that cells could be grown in blood plasma and that cancerous cells survive better than cells derived from non-cancerous tissues. Today, it is standard practice to use cell lines that have been transformed by a virus, a chemical, or cells that are derived from a carcinoma, for studies that require tissue culture. Cell culture is one of the easiest ways to propagate virus and study its replication, and is essential for the propagation of bornavirus, as it is highly cell-associated.

Primary cells that come directly from an animal tissue, naturally have a built in limit to the number of times that they can divide, and eventually will stop dividing. This process is called replicative cell senescence(2) and is a genetically determined phase(1). Normal cells stop dividing when they differentiate, in response to stress, otherwise DNA damage can occur. There is an arrest cycle, which is a checkpoint that allows cells to repair any damaged DNA. If the DNA cannot be repaired, the cell commits suicide by a process called apoptosis (2).

If left unchecked, cells can acquire a mutation and lose the ability to respond to DNA damage, which may turn into cancer immortalized cells. They then will continue to multiply and increase the number of chromosome abnormalities(2). Chemical carcinogens that cause local changes in the nucleotide sequence of DNA also induce cancer. For example, radiation causes chromosomes to break and translocate, and UV light induces specific DNA base alterations, such as thymidine dimerization (2). If an animal inherited a defect in a DNA

repair mechanism in the cell, the cell becomes predisposed to develop cancer. In order for cancer to develop, several mutations in DNA are needed to induce cell proliferation without restraint.

In order to study viruses and cells in culture scientists require cells that continuously divide, or have a significantly extended life before senescence occurs. There are many strategies that are used to prevent the cells from reaching senescence. Induction of cancer and growing a cell line from a tumor are the most commonly utilized techniques. Despite these well-known methods, there have been no continuously replicating cell lines derived from a psitticine. It remains unknown if a psitticine cell line can be transformed with a cancer causing viral protein, such as the SV40 large T-antigen, we used a known carcinogenic chemical to attempt cellular transformation in a primary psitticine cells.

The carcinogen utilized in this study, 3-methylcholanthrene (3-MCA) is a polycyclic aromatic hydrocarbon that is known to induce carcinogenic changes in cells. It has been shown to induce tumors when injected into living rats (5) as well as in culture using Swiss mouse embryo fibroblasts(2). For this reason, it has been used to transform several cell types to generate multiple cell lines, including those derived from avian species (6). Similarly, we will utilize 3-MCA to induce tumors in cells derived from cockatiel or parrot embryos for the creation of continuous, tumorigenic cell lines. These newly derived cell lines initially will be used to study bornavirus infection mechanisms.

CHAPTER II

METHODS

Primary Embryo fibroblast cells were from nine day old embryos as described below. The cells were then plated and grown. The cells were treated with 3-MCA for forty eight hours when they were 50% confluent. After treatment cells were watched for morphological changes, which upon occurrences were removed and plated in a new well. If the cells regrew upon passage they were continued to be passaged until they were deemed immortal.

Collection of embryo fibroblast

The eggs were incubated for nine days and placed in 4 degrees for thirty minutes to terminate. I then sterilized the eggs with 70% Ethanol. The tops of the eggs were cut away and the embryo removed. The embryos were decapitated, extremities and intestines removed. The heads were given to Joe Guo for PCR testing for borna virus. Embryos were then placed into a trypsinizing flask. They were washed three times with PBS removing all red blood cells. 25 ml of trypsin were added with a stir bar and placed on a stir plate for 6 min. The supernatant was removed and poured through nine layers of cheese cloth into a beaker. I repeated the trypsinization twice. The cells were spun down at 1000 rpm for 5 min. I then discarded of the supernatant and resuspended the pelleted cells in multiple one ml freezing medium (10% DMSO+51% FBS+ 39% Media). To cryopreseve the cells, they were placed into a freezing container (Mr. Frosty) and transferred to the -80º Cfreezer. After three days I moved them to a cardboard box in -80º C freezer.

Chemical treatment of the cells

The primary cells isolated directly from the duck or cockatiel embryo were plated in a 48-well culture plate at ~50% confluency. When the cells grew to 50% confluency and appeard healthy, ie were adhesive and stretched out well, they were starved for fetal bovine serum and growth factors in phosphate buffered saline (PBS) for one hour prior to treatment with 3-Methylcholanthrene. The cells were treated with various concentrations of 3-methylcholanthrene initially dissolved in acetone.

I used a 48 well culture plate so four different concentrations of carcinogen could be evaluated in six wells each, specifically .25ug/ml, .5ug/ml, 1 ug/ml, and 1.5 ug/ml of 3MCA. In addition, six concentrations were used in triplicate: 2 ug/ml, 3 ug/ml, 4 ug/ml, 5 ug/ml, 6 ug/ml, 7 ug/ml Three wells of cells received 1.424 ul of acetone only and three wells left untreated as controls (Table 1). All of the wells received 300 ul of media with 20% FBS to facilitate the solubility of the highly organic nature of the 3-MCA and to provide nutrients for the cells.

Cells were treated with 3-MCA for forty-five hours and then washed twice with PBS to remove any residual chemical. The cells received 1 ml of fresh media per well and the media changed every day for four days to make sure all carcinogen had been removed. Subsequently, the media was changed once every three days subsequently.

Table 1. Forty eight-well plate 3MCA concentrations

Control with acetone	6ug/ml	4ug/ml	2ug/ml	1.5ug/ml	1ug/ml	.5ug/ml	.25ug/ml
Control with acetone	6ug/ml	4ug/ml	2ug/ml	1.5ug/ml	1ug/ml	.5ug/ml	.25ug/ml
Control without acetone	7ug/ml	5ug/ml	3ug/ml	1.5ug/ml	1ug/ml	.5ug/ml	.25ug/ml
Control without acetone	7ug/ml	5ug/ml	3ug/ml	1.5ug/ml	1ug/ml	.5ug/ml	.25ug/ml
Control without acetone	7ug/ml	5ug/ml	3ug/ml	1.5ug/ml	1ug/ml	.5ug/ml	.25ug/ml

Monitoring cell changes

An Olympus IX70 inverted microscope equipped with a SPOT digital camera was used to visualize and take pictures of the cells. Pictures were taken before the cells were treated and every 12 hours after treatment, until the 45th hour. The carcinogen was removed and then pictures were taken every 12 hours for the next 2 days. After this, pictures were taken once every 24 hours. The purpose of taking pictures was to note any changes in morphological structure, cellular arrangement, and/or the formation of large giant cells and to maintain a permanent record of these changes.

Once morphological changes were noted or there was formation of cellular aggregates, the cells were drawn up into a Pasteur pipet and plated into a small 96 well plate. The cells continued to be expanded and the passages counted until they were considered to be an immortal cell line.

Medium

The medium that was used consisted of a mix of 38.75% DMEM Dulbeccos Modified Eagle's Medium (DMEM), 38.5% Minimum Essential Medium (MEM), 20% FBS fetal bovine serum, 0.5 % of non-essential amino acids (NEAA), 0.5 % L-glutamine, 0.5% sodium pyruvate, 1.0% antibiotic-antimycotic. This mixture was used so the cells were exposed to the high glucose of the DMEM as well as the nutrients from the MEM for maximal survival.

Reconstituting the 3-MCA in acetone

The 3-MCA purchased from Ultra scientific (product number RAH-041 10MG) was diluted in 2 ml of acetone to reconstitute it. The final volume of stock solution was 5MG/ml. When adding the chemical to the cells I took 14ul of the solution and added it to a total volume of 10 ml of media. From this stock solution .1428 ml was added to 3.8572 ml of media to make a final volume of 4 ml. This protocol was repeated for four more additional concentrations of chemical with an increase in 0.1428 ml of stock solution per six wells for concentration up to .8572 ml. This resulted in six triplets of different concentrations. The chemical was

added at a final volume of 2 ml. There are six different concentrations of chemicals with an increase in .2858 ml of stock solution per triplet from 0 .5714 ml up to 1 ml.

Passaging the cells

The cells were passaged using 0.25% trypsin+EDTA to release adhesion from the plastic wells. The cells were washed with PBS twice, washed with trypsin, and incubated in trypsin for five to ten min or until the cells began to lift off of the plate. Medium with FBS was added to the trypsin/cell mixture to quench the enzymatic reaction, and the mixture centrifuged at 2000 rpm at 25°C for ten min. to pellet the cells. The supernatant was removed and the cells were resuspended in media and plated in the appropriate flasks or culture plates.

CHAPTER III

RESULTS

This project has been proven difficult for many reasons. There currently are no Cockatiel cell lines and very few duck cell lines indicating the resistance of the cell to grow in culture. Tissue culture requires absolute sterile technique and even while utilizing good sterile technique, problems can still arise. There have been problems with fungus as well as bacteria contamination in the lab cultures. These problems arise not only from technique but improper maintenance of the tissue culture incubators and water baths. There have been problems deciding on the optimal transformation protocol since there is so little known about avian cells. Without knowing the type of surface receptors that are present and plasma membrane composition, we did not know which plasmid to use for immortalization and did not want to go on a "fishing expedition." Chemical treatment was decided to be the best technique to use for immortalization since it bypassed receptors and could enter cells despite the lipid composition of the membranes.

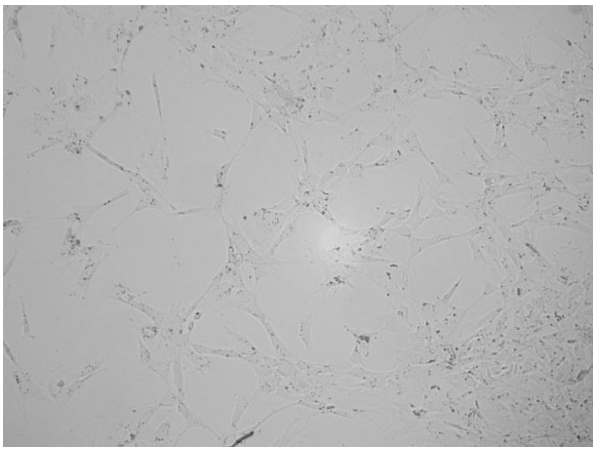

Figure 1. Control DEF

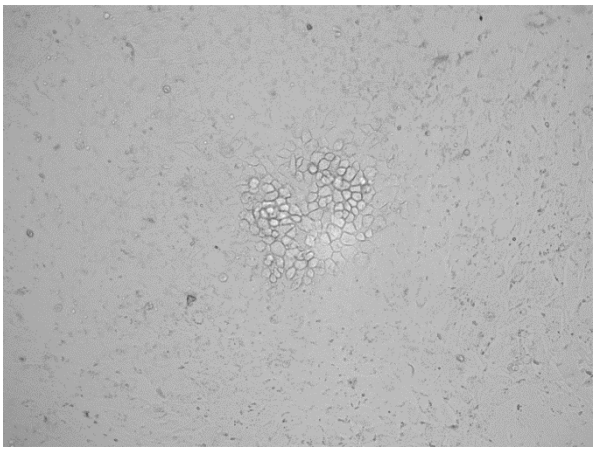

Figure 2. 3-MCA .5ug/ml 3mca foci

Figure 1 shows the elongated cells of the untransformed control, while Figure 2 shows a foci of cuboidal transformed cells.

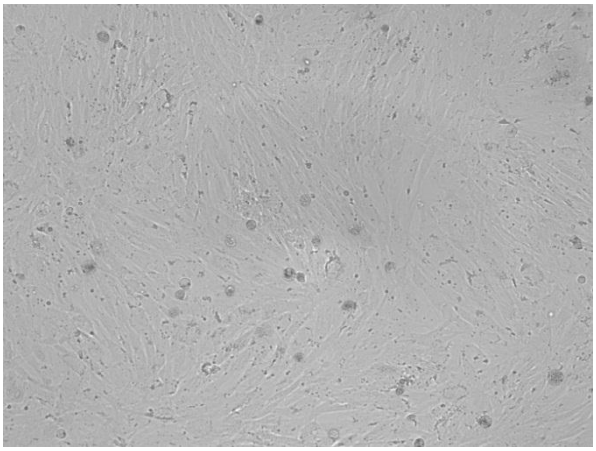

Figure 3. DEF control

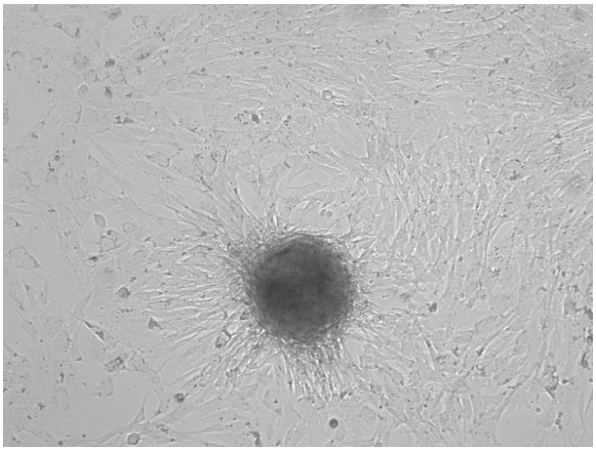

Figure 4. DEF 7ug/ml 3-MCA foci

Figure 3 shows a confluent layer of cells, while Figure 4 shows a dense foci of cells that had stacked ontop of eachother.

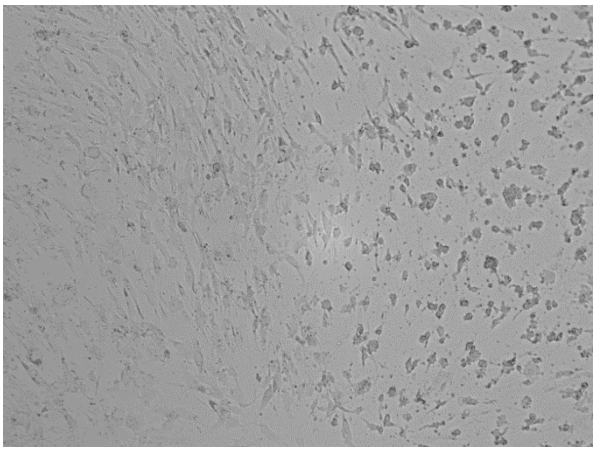

Figure 5. DEF 2.0 ug/ml 3-MCA

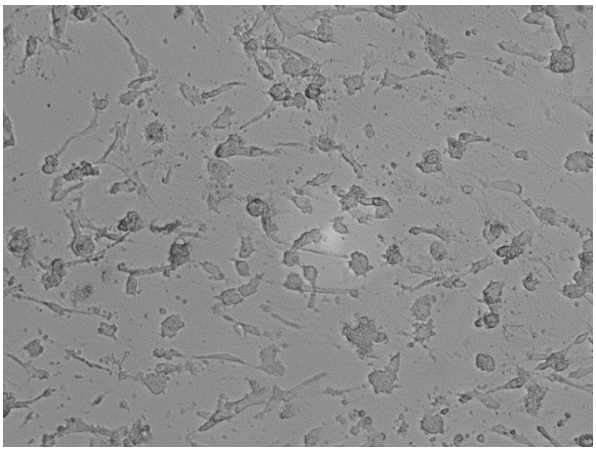

Figure 6. 20x of right field of Figure 5

Figure 5 is a split view of one well where the normal cells are on the left side of the picture and the transformed cells on the right. Figure 6 is a 20x picture of the cells from the right field of Figure 5 Note the circular and spindle shape.

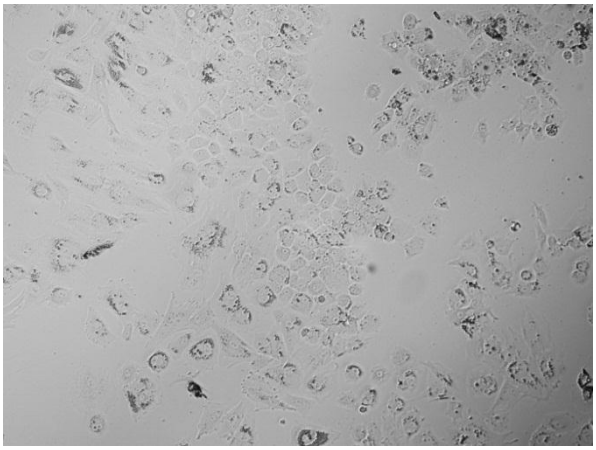

Figure 7. CTEF 0.25ug/ml 3-MCA

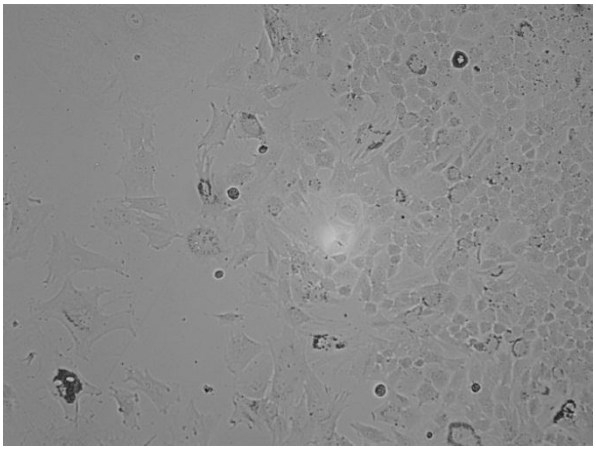

Figure 8. CTEF 0.5ug/ml 3-MCA

Figure 7 and 8 shows treated Cockatiel embryo fibroblast that started to grow and continued to after most controls had died. The controls retained their fibroblastic appearance, elongated and stretched out, while foci of circular cells began to show up. The foci started out small with just a few cells and then began to grow outward.

Plating tumor cells from an African Grey parrot

We first decided to try to expand a preexisting carcinoma from a parrot. The cells were already immortal, making them the perfect candidate for an immortalized cell line. An African grey parrot had been diagnosed with simple squamous cell carcinoma and it was biopsied. The tumor biopsy was sliced into pieces, plated with growth media and incubated. The tissue mostly floated in the media and trypsinizing the tissue two days later failed to provide different results. The tissues might have produced different results had we homogenized or trypsinized the tissue from the beginning. No cell line was established form this method.

Harvesting DEF

In order to obtain primary cells for chemical immortalization, the cells were harvested from embryos of nine day old fertile duck eggs. Due to past contamination of DEF with borna virus, the heads of the embryos were removed and tested for BVD using PCR. The results would reveal if the embryo were already infected. For this project we purchased certified clean duck eggs from the FEDS. PCR testing revealed that two of the first twelve embryos were infected despite being certified clean. After testing four more batches of duck eggs

from four different providers, one of the four batches were found to have two bornavirus-positive heads. This alludes to the idea that borna virus is passed vertically from the duck to the egg, but further testing is needed to verify this. I also suggest that bornavirus is much more common than realized.

Harvesting CTEF

The process of harvesting primary cockatiel cells was very successful. Four eggs were used and all four heads tested PCR negative for borna virus. From them ten vials of CTEF were made and frozen. Each vial successfully plated onto a single T25 flask with 70% confluency after 24 hours. However, the CTEF would only passage three times before going into senescence. This drastically reduced the amount of cockatiel cells available to work with.

Treating with chemical

In order to attempt to immortalize primary avian cells using a chemical, 3-MCA was used based on success in the literature (7). The chemical concentration used in the beginning ranged from 0.5ug/ml to 3.0ug/ml. The concentration range later changed from .25ug/ml to 7ug/ml. Upon treatment of the cells with 3-MCA both the DEF and CTEF showed foci with morphological changes as well as outgrowth from the foci. The foci changed form a fibroblast-like appearance to a cuboidal-like appearance (normal Figure 1and 3 and cuboidal figure 2, 4, 5 and 6). Morphological changes occurred at all ranges, but averaged around 5ug/ml. To date, a large number of cells for long term culture have not been successfully

immortalized. A wide variety of methods to transfer the foci have been used, but senescence appears to occur after one or more passages to a new plate.

The first plate treated with 3MCA was infected with a fungus early and no results were obtained. The second 3MCA treatment was two 24 well plates with one morphological change per plate. Instead of trypsin a pipet was used to suck the morphologically changed cells off the plate for transfer and this did not prove to be effective.

The methods attempted did not work as well as we had hoped. CTEF were then starved of nutrients (only given PBS for one hour) to force them to take up more chemical. Using this method, we had a drastic increase in the number of foci and morphological changes following chemical treatment in two 48 well-plates Figure 7and 8. The concentration of chemical ranged from 0.25ug/ml to 6mg/ml of 3-MCA and there was foci present but were discarded due to bacterial contamination.

Two more CTEF confluent 48 well plates were treated and five wells of one plate and seventeen wells from the other plate had foci of morphological changes. They were transferred by scraping the cells with a pipet tip and this was unsuccessful. None of the transferred cell continued to divide.

Due to the problems with fungal and bacterial contamination using the 24 and 48 well plates a switch was made to T25 flask. This cut down on infection but the cells stopped growing

after being split to a T75 flask. However, recently the cells transferred more efficiently in the T25 and T75 flask than the 24 and 48 plates. The flasks are being further evaluated to see if they will continue to divide or go into senescence.

Endotoxin test

Due to the assumption that endotoxins may be causing harmful effects on the primary cells the glassware in the lab was tested for endotoxin. We use glassware to store media in and as well as well as trypsin. We found some of our glassware and reagents contained endotoxin. Therefore, we implemented a protocol to rid the endotoxin from the glassware that has been tested and works. However, the lack of endotoxin effects on the cells remains unknown.

Chemical problems

Our next challenge was the stock solution of 3-MCA used for immortalization began to fall out of solution (acetone). Upon heating, the 3-MCA would dissolve but precipitated when the solution was cooled. Consequently, we switched to DMSO, a chemical that is widely used in tissue culture, to dissolve the 3-MCA.New chemical was purchased and then was dissolved DMSO at a concentration of 7mg/ ml.

Picture problems

Most of my earlier pictures were deleted from a scratch drive these contained a lot of the pictures from early in the project. We are in the process of having someone try to retrieve them.

CHAPTER IV

SUMMARY AND CONCLUSIONS

In this study we show that upon treatment with the known carcinogen, 3-MCA, morphological changes are induced in the primary avian cells Figure 1 through Figure 8. Some of the cells changed from being stretched out to a round/cuboidal appearance. Other cells rounded up and had a spindle like appearance. In addition we see increased cellular growth as well as stacking of the cells into dense foci as in Figure 4.

The Cockatiel cells are more sensitive to the 3-MCA than the duck cells as evidenced by the occurrence of more numerous morphological changes; however they did not transfer well. Whether this is technique or a property of the duck embryo is unknown. The CTEF cells became cuboidal upon treatment Figure 7 and 8 in small foci and continued to grow outward if left alone, even after control cells had died. When being passaged the cells were much more adherent to the plate and trypsinizing did not work efficiently. If media was placed back into the trypsinized well in some of the cells that had not transferred would continue to grow outward. Once transferred to a new well they would stop dividing. A feeder layer of cells has been previously shown to aid in the growth of cells of interest. A feeder layer is a layer of cells that conditions the media, releasing growth factors that are beneficial for cell growth. Cells of interest are cultured with the feeder layer and continue to grow while the feeder layers die off. Some feeder layers are already chemically induced into senescence so

they will not passage with the cells of interest. A feeder layer could potentially be beneficial in aiding the growth of the morphologically changed CTEF and DEF cells when passaging.

One plate of treated DEF cells resembled transformed cells that was presented in Moscovici et al paper (6) using transformed quail cells. The cells were cultured from tumors induced by 3-MCA injections. They were spindle-shaped and had round cells but did not passage Figure 5. DEF treated cells that formed a dense foci Figure 4 resembled the type III foci presented in Mukherjee et al paper (7) who used 3-MCA to transform murine embryonic fibroblast. The foci were multilayer with cells crossing in different directions instead of normal stretched out cells. The dense foci was a much more common morphological change than the spindle shape

There have been many problems along the way due to contamination and not getting the morphologically changed cells to transfer into a new plate. The morphological changed cells that have passaged still need to be evaluated and grown to see if they will continue to divide or go into senescence.

Contamination comes from a variety of reasons. Your incubator could become contaminated from putting in or taking out culture flasks. The water bath gets exposed to the environment as well as contaminated bottles. The incubators and water baths need to be sterilized and the

water changed weekly. Well plates are not protected by a filter from the environment, while traveling to take picture to or at microscope they can easily become contaminated. Tissue can come be contaminated with bacteria and fungus and contaminate cultures derived from them.

Using a chemical to treat the cells raises the question of whether there is another way to immortalize avian cells? The answer is that there are other ways to immortalize a cell line such as using viral vectors or plasmids, but very little is known about the proteins expressed in duck and especially cockatiel cells.

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